Physiological diversity of the first filamentous fungi isolated from the hypersaline dead sea

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A number of filamentous fungi isolated from different sites and depths of the hypersaline Dead Sea, including the new species *Gymnascella marismortui*, were tested under different temperature and salinity regimes on agar plates for colony growth, enzyme production (amylase, caseinase, cellulase, urease) and degradation of synthetic dyes. Whereas *Ulocladium chlamydosporum* (mitosporic fungi) appeared poorly adapted to temperatures and salinities of the Dead Sea, *G. marismortui* (Ascomycetes) was found to be much better adapted. The cosmopolitan *Penicillium westlingii* (Mitosporic fungi) strains showed little effect of salinity and temperature on growth. Enzyme production and the number of dyes being degraded by each of the strains generally decreased with increasing salinity.

Key words: Dead Sea, dye degradation, enzymes, filamentous fungi, salinity, temperature.

Introduction

The Dead Sea, located at the lowest altitude on Earth (present level: 412 m below mean sea level) is a harsh environment even for those microorganisms best adapted to life at high salt concentrations. The peculiar ionic composition of its water, with its high concentration of the divalent cations magnesium and calcium, is highly inhibitory even to the most halophilic and halotolerant microorganisms. Since the beginning of this century the water balance of the Dead Sea has been negative (Gavrieli *et al.*, 1999). The decrease in water level, due in part to climatic changes and intensified by the diversion of fresh water from the Sea of Galilee and the Jordan river, has resulted in an increase in

salinity of the upper water layers. Massive amounts of NaCl have precipitated from the water column to the lake bottom as halite crystals (Gavrieli, 1997). The precipitation of halite has caused an additional increase in the already extremely high ratio of divalent to monovalent cations of the water. The mean values for the ionic concentrations in 1996 were (in mol/L): Mg^{2+}, 1.887; Na^{+}, 1.594; Ca^{2+}, 0.436; K^{+}, 0.199; Cl^{-}, 6.335; Br^{-}, 0.068, and SO_4^{2-}, 0.005. The lake is now holomictic, but meromictic regimes have occurred from 1979-1982 and from 1992-1995 as a result of massive inflow of fresh water during unusually rainy winters, with the formation of a pycnocline at a depth varying between 5 and 15 m.

Since the first reports on the existence of an indigenous microflora in the Dead Sea in the 1930s (Wilkansky, 1936; Elazari-Volcani, 1940; Volcani, 1944), our knowledge of the microbiology of the lake has greatly increased. The first quantitative determinations of the algal and bacterial community size in the lake were performed in 1963-1964 (Kaplan and Friedmann, 1970), but only from 1980 onwards have the temporal and spatial distribution of the microbial communities in the lake been systematically measured. Our understanding of the microbial ecology of the Dead Sea has been summarized in a number of review papers (Nissenbaum, 1975; Oren, 1988, 1997, 2000). *Dunaliella* sp. is the only primary producer in the lake. Algal blooms are not an annually recurring phenomenon, but they develop only following exceptionally rainy winters such as occurred in 1979-1980 and 1991-1992 (Oren and Shilo, 1982; Oren, 1993, 1999, 2000; Oren *et al.*, 1995). For *Dunaliella* to grow in the lake, the upper water layers must become diluted by at least 10% with fresh water, and phosphate, being the limiting nutrient, must be available (Oren and Shilo, 1985).

Development of *Dunaliella* blooms is followed by massive growth of halophilic Archaea of the family *Halobacteriaceae*. These Archaea were found in numbers of up to 1.9×10^7/mL in 1980 and up to 3.5×10^7/mL in 1992 (Oren, 1983, 1993, 1999; Oren and Gurevich, 1995). At the height of the blooms they impart a reddish color to the waters of the lake. When a new holomictic episode starts, the remainder of the biota in the epilimnion becomes distributed evenly over the entire water column (Oren, 1985, 1999, 2000; Oren and Anati, 1996). Cell densities and heterotrophic activities during the holomictic periods were found to be extremely low (Oren, 1992).

Until recently prokaryotic microorganisms were considered to be the only decomposers in the Dead Sea (Oren, 1988, 1997). However, it has recently been found that fungi, long neglected as a component of the food web in the Dead Sea and in other hypersaline environments as well, may also play a role (Buchalo *et al.*, 1998a).

A variety of fungi have been isolated from soils collected in the Dead Sea area, including hypersaline desert soils and soils collected from the oases of Einot Zuqim and Ein Gedi (Steiman *et al.*, 1994, 1995, 1996, 1997, Guiraud *et al.*, 1995, 1997; Volz and Wasser, 1995; Volz *et al.*, 1996). The fungal community found very much resembled that of common soils, and most species recovered have a worldwide distribution. A few novel species, however, were encountered in soil around the Dead Sea as well: a new species of the genus *Bipolaris*, described as *Bipolaris israeli* (Steiman *et al.*, 1996), two new species of *Aspergillus* (*A. homomorphus* and *A. pseudo-heteromorphus*) (Steiman *et al.*, 1994), and *Exserohilum sodomii*, a strain that also grows in hyperosmotic medium (8% NaCl, 3% sucrose and 5% malt extract) (Guiraud *et al.*, 1997).

The first isolation of a fungus from the Dead Sea water column was reported by Kritzman (1973), who isolated an osmophilic yeast from the lake that grew in a medium containing 15% glucose + 12% salt. No further details were given, and unfortunately no cultures were preserved.

During the years 1995-1997 a variety of fungi were isolated from the Dead Sea, both from surface water at the shore and in the center of the lake and from deep water samples. At least 26 species were found, most of them belonging to the ascomycetes, but mitosporic fungi and zygomycetes were encountered as well (Buchalo *et al.*, 1998a,b, 1999, 2000). Most species identified were common soil fungi, not well adapted to life at high salt concentrations. Of the species isolated by our group from waters of the Dead Sea, *Aspergillus niger*, *Cladosporium cladosporioides*, *Thielavia terricola* and *Ulocladium chartarum* had been isolated also by Volz and Wasser (1995) from soil around the lake. One of the isolates, described as a new species *Gymnascella marismortui* (Ascomycetes), is a true halophile that grows well in media containing 50% Dead Sea water and even higher. Optimal growth was observed in media containing 10-30% (by volume) of Dead Sea water, or in 0.5-2 M NaCl. Other isolates of halotolerant fungi from the Dead Sea able to grow in 50% Dead Sea water media were *Ulocladium chlamydosporum* (growing best at 3-15% NaCl at 26 C) and *Penicillium westlingii* (Buchalo *et al.*, 1998a,b, 1999, 2000; Molitoris *et al.*, 1998). Whether all these fungi were present in the Dead Sea water as vegetative hyphae or only as spores cannot yet be ascertained.

The purpose of this study was to investigate colonial growth and enzyme activities (amylase, caseinase, cellulase, urease) for selected fungal isolates of the Dead Sea at different salinities and temperatures as indication of their adaptation to the extreme environment of the Dead Sea. In addition, decolorization of 11 dyes of 4 chemical groups as a model system to test the potential degradation of polluting agents was determined on agar plates at different salinities and temperatures.

Materials and methods

Fungal strains

The following strains of filamentous fungi isolated from surface and deep waters of the Dead Sea (Buchalo *et al.*, 1998a,b, 1999, 2000) were used (Table 1): *Aspergillus phoenicis*, *Chaetomium nigricolor*, *Emericella nidulans*, *Gymnascella marismortui* (3 strains), *Paecilomyces farinosus*, *Penicillium variabile*, *P. westlingii* (2 strains) (all Ascomycetes), and *Acremonium* sp., *Stachybotrys chartarum*, and *Ulocladium chlamydosporum* (Mitosporic fungi).

Media

For propagation of cultures, growth studies and as basic medium for enzyme and dye decolorization tests we used GPY medium (0.1% glucose, 0.05% peptone, 0.01% yeast extract, and 1.6% agar, pH 6 before autoclaving) (Molitoris and Schaumann, 1986; Rohrmann *et al.*, 1992). Media were prepared in deionized water (indicated below as I), authentic Dead Sea water (D), dissolved Dead Sea salt (Schaeben, Germany) (S), commercial artificial seawater (RILA) of different salinities as indicated below (R), and artificial seawater (A).

Cultivation and determination of growth

Growth was determined as colony diameter in GPY Petri dishes (9 cm diam.), twice weekly, for up to 7 weeks at temperatures of 15, 22, 27, and 35 C, and salinities of 0% (I), 3.5% (A3), 17.5% (A17), 26% (A26) following Buchalo *et al.* (1998a,b, 1999, 2000). For comparison, the temperature of the Dead Sea surface water varies from about 18 C in winter to up to 35-36 C in mid-summer, while the whole range of salinities from fresh water to undiluted Dead Sea brines (around 340g dissolved salts per liter) may be encountered in areas in which freshwater enters the lake (e.g. the mouth of the Jordan River, freshwater springs such as Einot Zuqim).

Enzyme tests

Enzyme activities were determined in GPA Petri plates (amylase, caseinase, cellulase) following Molitoris and Schaumann (1986) and Rohrmann *et al.* (1992), and for urease in GPY agar slants following Christensen (1946). Amylase activity was detected as blue pigment after flooding the plates with Lugol iodine solution, caseinase and cellulase activity as extent of substrate clearing. Urease activity was followed by the change of the bromocresol purple pH indicator from yellow to red. Five strains were used as indicated in Table 1. Assays were performed at 15, 22, 27, and 35 C and at salinities of 0% (I), 3.5% (A3), 17.5% (A 17) and 26% (A26), and the results were read twice weekly for

Table 1. Dead Sea fungi isolated and investigated for growth, enzyme activity and dye decolorization.

Fungus	Growth (3 temperatures 3 salinities 5 water types)	Enzymes (4 temperatures 4 salinities 2 water types)	Dye-degradation (1 temperature 3 salinities 2 water types)	Ecotype (temperature, salinity)
Zygomycetes				
Absidia glauca				
Ascomycetes				
Aspergillus caespitosus				Thermotolerant
A. carneus				Thermotolerant
A. fumigatus				
A. niger				Thermotolerant
A. phoenicis			1 strain	
A. terreus				
A. ustus				
Chaetomium aureum				
Ch. flavigenum				
Ch. funiculosum				Thermotolerant
Ch. nigricolor			1 strain	
Cladosporium cladosporioides				
Cl. macrocarpum				
Emericella nidulans			1 strain	
Eurotium amstelodami				
E. herbariorum				
**Gymnascella marismortui*	3 strains	3 strains	1 strain	Halophilic + thermotolerant
Paecilomyces farinosus			1 strain	Thermotolerant
Penicillium variabile			1 strain	Thermotolerant
**P. westlingii*	2 strains	1 strain	1 strain	Halotolerant + thermotolerant
Thielavia terricola				Thermotolerant
Mitosporic taxa				
Acremonium sp.			1 strain	
Ac. persicinum				Thermotolerant
Stachybotrys chartarum			1 strain	Thermotolerant
**Ulocladium chlamydosporum*	1 strain	1 strain	1 strain	Not adapted to high salinity
13 genera, 26 species	6 strains	5 strains	10 strains	

* Isolations from surface water of the Dead Sea near Einot Zuqim, all other isolations from different depths of the Dead Sea near Ein Gedi (Ref.: Buchalo *et al.*, 1998a,b).

up to 7 weeks. Enzyme activity was assessed semiquantitatively using a scale of 0 = none, 1 = weak, 2 = moderate, and 3 = strong.

Dye decolorization

Dye decolorization was followed at 22 C and salinities of 0% (I), 3.5% (A3) and 17.5% (A17) on GPY Petri plates, using 10 strains as indicated in Tables 1 and 2. All dyes were used at a final concentration in the agar medium of 200 ppm. We included two anthraquinone dyes (remazol brilliant blue R [RBBR] and disperse blue 3 [DB3]), three heterocyclic dyes (fluoresceine [FLC], methylene blue [MB], and bromophenol blue [BB]), three monazo dyes (methyl orange [MO], methyl red [MR], and reactive orange [RO]), and three diazo dyes (reactive black [RB], congo red [CR], and sudan black B [SBB]). Decolorizing activity was determined twice weekly for up to 7 weeks and assessed as 0 = none, 1 = weak, 2 = moderate, and 3 = strong/complete (Kurchenko *et al.*, 1998; Buchalo *et al.*, 1999).

Results and discussion

Growth

Colonial growth on Petri plates is an indication of the adaptations to the extreme conditions of the Dead Sea and was tested using six isolates obtained from this habitat (five ascomycetes, one mitosporic taxon) with different water types at salinities ranging from 0 to 26% and temperatures from 15 to 35 C (Table 1) (Molitoris *et al.*, 1998). Three different types of adaptation were observed.

The three isolates of the ascomycete *Gymnascella marismortui*, recently isolated and described from the Dead Sea (Buchalo *et al.*, 1998a), are obligately halophilic, as they did not grow in freshwater medium and showed increased growth with increasing salinity. This was particularly obvious at 35 C. The ionic composition of the medium had little effect on growth (Fig. 1a, Table 1). *Gymnascella marismortui* therefore might be well adapted to life in the Dead Sea. The ascomycete *Penicillium westlingii*, a cosmopolitan soil fungus, was relatively indifferent in its response of colonial growth to salinity and ionic composition of the medium and cultivation temperature as shown in Fig. 1b. This fungus therefore is halotolerant and thermotolerant (Table 1) and may thus be expected to be able to grow and propagate in the Dead Sea at locations of low salinity. *Ulocladium chlamydosporum*, a mitosporic fungus, represented the third group, which grew best in freshwater medium or at low salinities; its growth decreased with increased salinity and temperature. This reaction type was independent of the ionic composition of the media (Fig. 1c). *Ulocladium chlamydosporum* is not well adapted to the high salinity and temperature of the

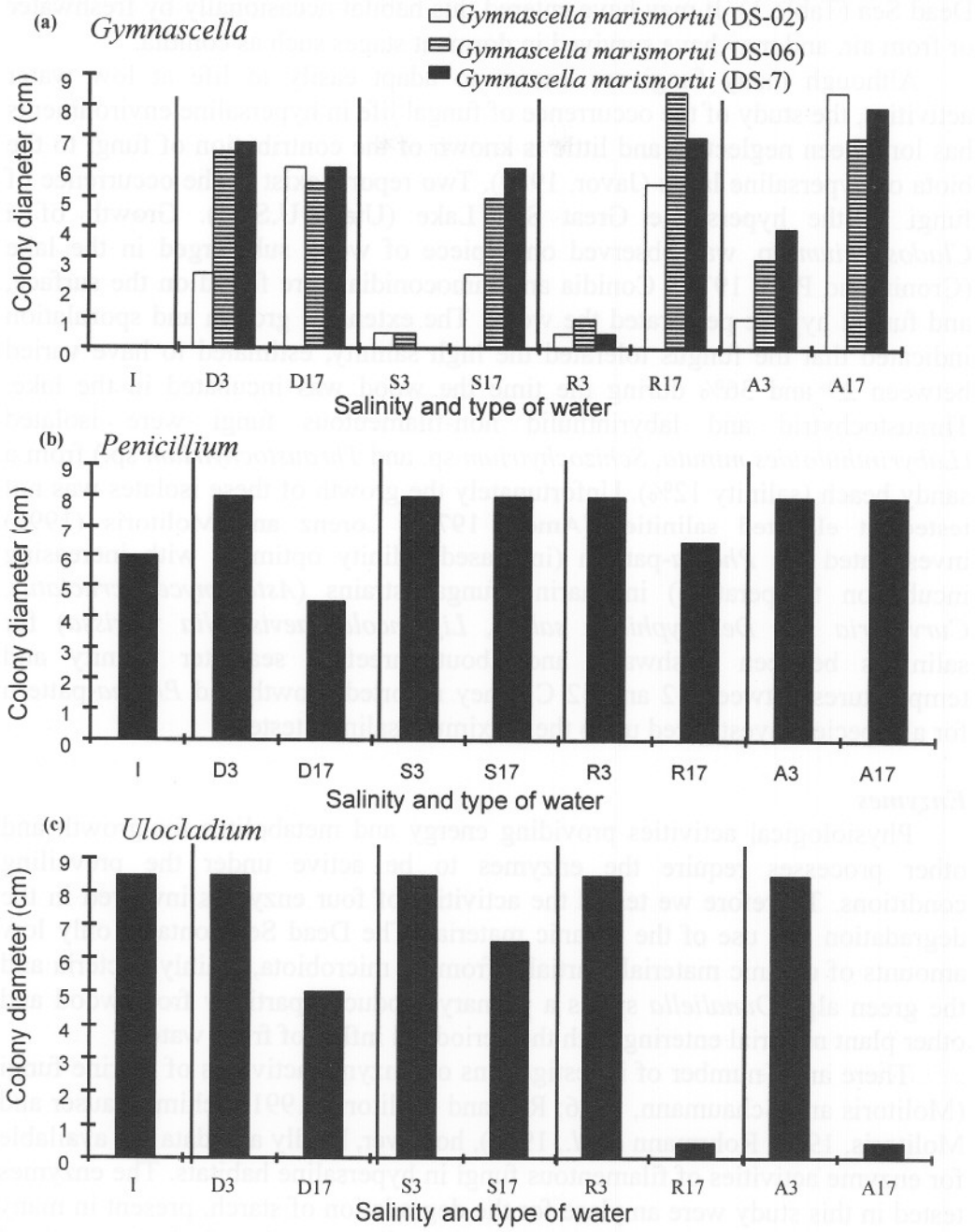

Fig. 1. Growth of fungal Dead Sea isolations on agar plates depending on salinity and type of water (35 C, 7 weeks). **a.** *Gymnascella marismortui*; **b.** *Penicillium westlingii*; **c.** *Ulocladium chlamydosporum*.

Dead Sea (Table 1). It may have entered this habitat occasionally by freshwater or from air, and may have survived in dormant stages such as conidia.

Although many fungi are known to adapt easily to life at low water activities, the study of the occurrence of fungal life in hypersaline environments has long been neglected, and little is known of the contribution of fungi to the biota of hypersaline lakes (Javor, 1989). Two reports exist of the occurrence of fungi in the hypersaline Great Salt Lake (Utah, U.S.A.). Growth of a *Cladosporium* sp. was observed on a piece of wood submerged in the lake (Cronin and Post, 1977). Conidia and ramoconidia were found on the surface, and fungal hyphae penetrated the wood. The extensive growth and sporulation indicated that the fungus tolerated the high salinity, estimated to have varied between 29 and 36% during the time the wood was incubated in the lake. Thraustochytrid and labyrinthulid non-filamentous fungi were isolated (*Labyrinthuloides minuta*, *Schizochytrium* sp. and *Thraustochytrium* sp.) from a sandy beach (salinity 12%). Unfortunately the growth of these isolates was not tested at elevated salinities (Amon, 1978). Lorenz and Molitoris (1992) investigated the *Phoma*-pattern (increased salinity optimum with increasing incubation temperature) in marine fungal strains (*Asteromyces cruciatus*, *Curvularia* sp., *Dendryphiella salina*, *Lignincola laevis*, *Nia vibrissa*) for salinities between freshwater and about threefold seawater salinity and temperatures between 12 and 42 C. They reported growth and *Phoma*-pattern for all species investigated up to the maximum salinity tested.

Enzymes

Physiological activities providing energy and metabolites for growth and other processes require the enzymes to be active under the prevailing conditions. Therefore we tested the activities of four enzymes involved in the degradation and use of the organic material. The Dead Sea contains only low amounts of organic material, partially from its microbiota, mainly bacteria and the green alga *Dunaliella* sp. as a primary producer, partially from wood and other plant material entering with the periodical influx of fresh water.

There are a number of investigations on enzyme activities of marine fungi (Molitoris and Schaumann, 1986; Rau and Molitoris, 1991; Schimpfhauser and Molitoris, 1991; Rohrmann *et al.*, 1992), however, hardly any data are available for enzyme activities of filamentous fungi in hypersaline habitats. The enzymes tested in this study were amylase for the degradation of starch, present in many plant tissues; caseinase as an enzyme degrading protein of plant or animal origin; cellulase, the enzyme splitting cellulose, the major component of wood, representing the ubiquitous substrate for fungi, also in marine habitats; finally urease, an enzyme involved in the use of urea of animal origin as nitrogen

source. All enzyme activities were tested semiquantitatively in media of increasing salinity (0-26%) and over a range of temperatures (15-35 C) (Table 1).

Amylase activity was detected in three strains of *Gymnascella marismortui* and one strain each for *Penicillium westlingii* and *Ulocladium chlamydosporum*, both in freshwater medium and in artificial seawater media with salinities from 3 to 26% at temperatures from 15 to 35 C (Fig. 2). The highest activities were observed around 22 C, decreasing with increasing salinity, particularly at the highest temperatures. As judged by the reaction intensity on the agar media, caseinase showed generally the highest activities under all conditions tested with little effect of salinity and temperature (Fig. 3). The trend of decreasing activity with increasing salinity and temperature was for all strains similar for amylase and cellulase (Figs. 2, 4). Urease activity (Fig. 5) was high in *U. chlamydosporum* and *P. westlingii* and decreased only at the highest salinity (A26), whereas this enzyme showed maximum activity in the *Gymnascella* strains only at intermediate salinities (Kurchenko *et al.*, 1998; Buchalo *et al.*, 1999). Thus, enzyme activities generally decreased with increased salinity and temperature.

Dye degradation

Physiological activity of fungi can conveniently be assayed according to decolorization of synthetic dyes. Such dyes have been widely used as model compounds to monitor the self-cleaning capacity of waters. Being aromatic compounds, these dyes or their degradation products are often toxic, even carcinogenic, and are predominantly recalcitrant to degradation (Meyer, 1981; Davis *et al.*, 1994; Nigam *et al.*, 1996). Fungi already have been shown to be promising degraders of such dyes (Vyas and Molitoris, 1995; Sasek *et al.*, 1998).

We performed dye decolorization assays, using three mitosporic taxa and seven ascomycetes isolated from the Dead Sea (Tables 1, 2), eleven synthetic dyes of four chemical groups, performing the tests at 22 C and at three salinities (I, A3, A17) (Rawal *et al.*, 1998). All dyes and dye groups investigated showed a decrease in decolorization with increasing salinity, particularly between 3.5% (A3) and 17.5% (A17) salinity. This was less distinct for the monazo dyes. The only exception is methyl red, the decolorization of which was not affected by salinity. Heterocyclic dyes were decolorized best, followed by monazo and anthraquinone dyes; diazo dyes were decolorized least. The heterocyclic dye bromophenol blue and the monazo dye methyl red were decolorized best, sudan black, methylene blue and methyl orange were decolorized least.

Table 2. Influence of salinity on the decolorization of 11 dyes by fungi isolated from the Dead Sea.

Salinity	I (deionized water)												A_3 (3% salinity)											
Dye group	Anth		Hetero			MonA			DiA			Σ	Anth		Hetero			MonA			DiA			Σ
Dye	1	2	3	4	5	6	7	8	9	10	11		1	2	3	4	5	6	7	8	9	10	11	
Mitosporic taxa																								
U. chlamydosporum	-	2	2	-	1	-	-	-	-	2	-	4	-	3	3	-	2	-	2	-	-	-	-	4
S. chartarum	-	2	2	1	1	-	1	-	-	-	-	5	3	2	-	-	2	-	3	-	-	-	-	4
Acremonium sp.	-	1	-	-	-	-	2	-	-	1	-	3	-	3	-	-	2	-	3	-	-	-	-	3
Ascomycetes																								
E. nidulans	3	2	-	-	1	-	-	1	1	3	-	6	3	-	3	-	2	-	3	-	1	-	-	5
A. phoenicis	-	-	2	-	2	2	-	2	-	1	-	5	3	-	3	-	2	-	-	-	-	-	-	3
C. nigricans	-	2	2	-	2	-	2	-	-	-	-	4	-	3	-	-	2	-	1	-	2	-	-	4
P. westlingii	-	-	2	-	1	-	3	-	-	-	-	3	-	-	3	-	2	-	3	-	-	-	-	3
P. variabile	-	-	-	-	1	-	-	-	-	2	-	2	2	-	3	-	2	-	-	-	-	-	-	3
Pae. farinosus	-	1	-	-	-	-	2	-	-	2	-	3	-	-	-	-	2	-	-	-	-	-	-	1
G. marismortui	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	0
Σ dye degradations	1	6	5	1	7	1	5	2	1	6	0	35	4	4	5	0	9	0	6	0	2	0	0	30

Dye groups: Anth = anthraquinone, Hetero = heterocyclic, MonA = monazo, DiA = diazo.

Dyes: 1 = Remazol Brilliant Blue R; 2 = Disperse Blue 3; 3 = Fluoresceine; 4 = Methylene Blue; 5 = Bromophenol Blue; 6 = Methyl Orange; 7 = Methyl Red; 8 = Reactive Orange; 9 = Reactive Black; 10 = Congo Red; 11 = Sudan Black

* Dyes #5, 8, 9, 10 and 11 not tested at 17.5% salinity because of problems with solidification of the medium.

Decolourization: - = none, 1 = weak, 2 = good, 3 = strong/complete.

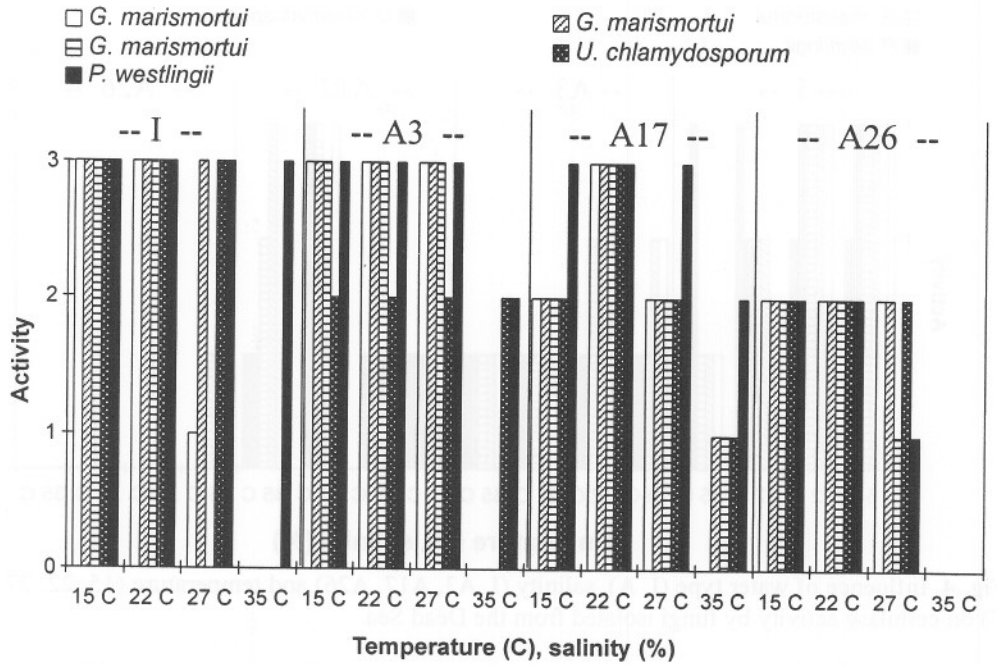

Fig. 2. Influence of water type (I, A), salinity (I, A3, A17, A26) and temperature (15, 22, 27, 35 C) on amylase activity by fungi isolated from the Dead Sea.

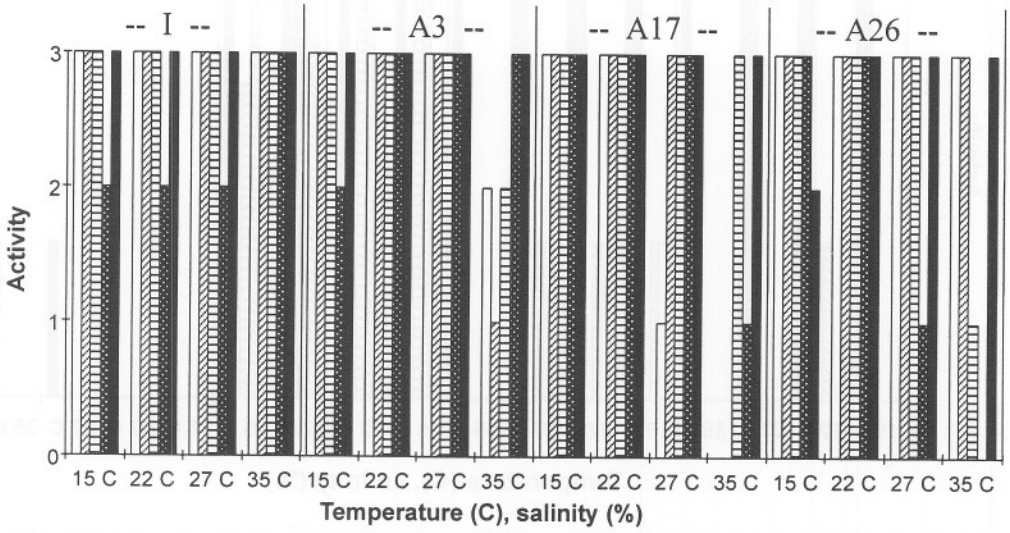

Fig. 3. Influence of water type (I, A), salinity (I, A3, A17, A26) and temperature (15, 22, 27, 35 C) on caseinase activity by fungi isolated from the Dead Sea.

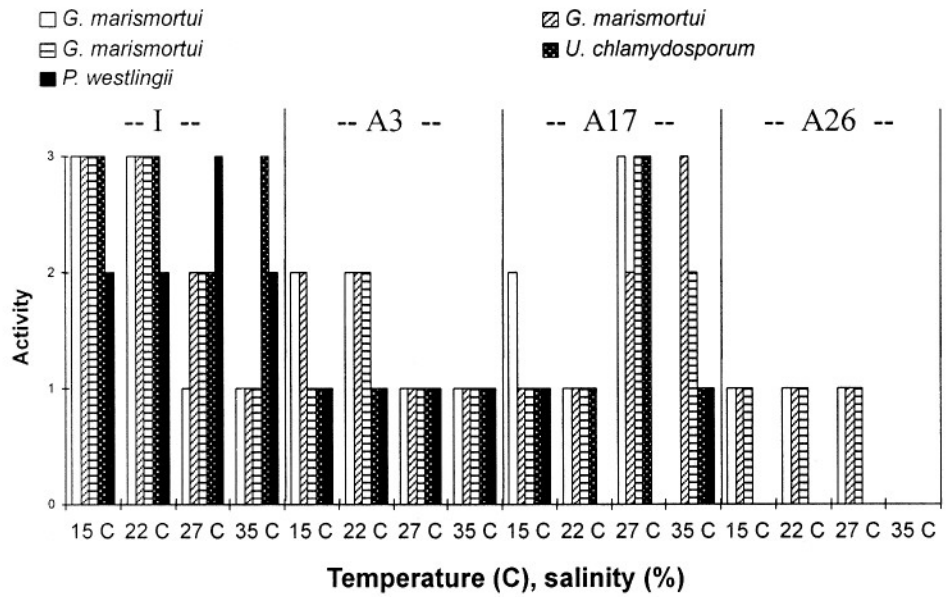

Fig. 4. Influence of water type (I, A), salinity (I, A3, A17, A26) and temperature (15, 22, 27, 35 C) on cellulase activity by fungi isolated from the Dead Sea.

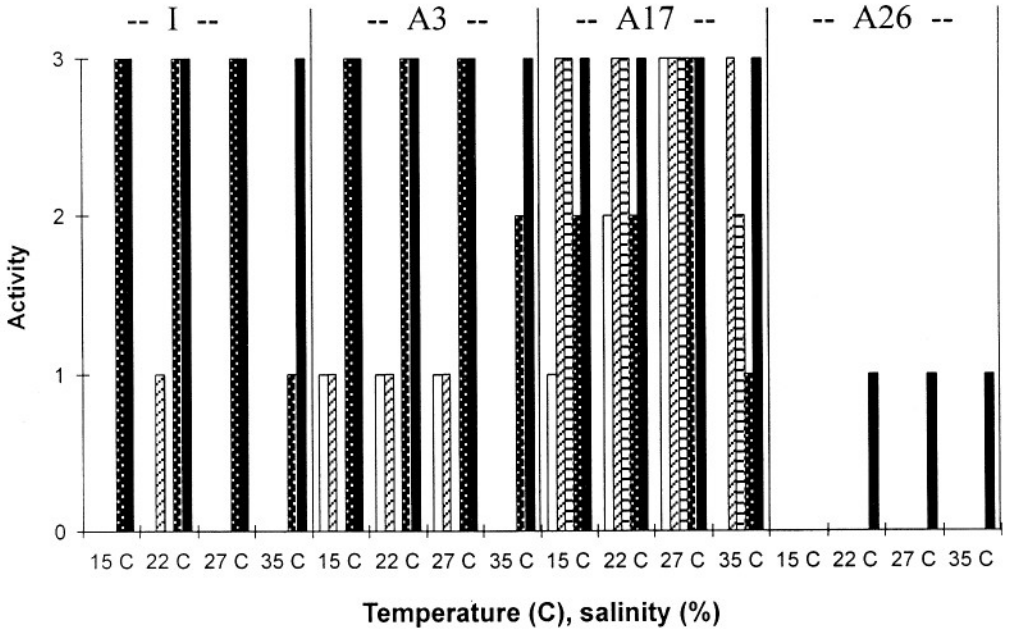

Fig. 5. Influence of water type (I, A), salinity (I, A3, A17, A26) and temperature (15, 22, 27, 35 C) on urease activity by fungi isolated from the Dead Sea.

No distinct correlation of dye decolorizing ability with fungal systematic groups (Mitosporic fungi/Ascomycetes) could be observed, dye decolorization rather seems to be an individual property for each species/dye combination. Among the Mitosporic taxa *Ulomyces chlamydosporum* was the best, and *Acremonium* sp. was the worst dye decolorizing fungus. Among the ascomycetes *Emericella nidulans* and *Aspergillus phoenicis* were the best decolorizers, and *Gymnascella marismortui* was not able to decolorize any dye at any salinity.

Since ligninolytic fungi have been shown to be among the best attackers of aromatic dyes (Vyas and Molitoris, 1995; Sasek *et al.*, 1998), future investigations should pay special attention to this group of fungi if isolated from this habitat. Our results on dye decolorization correspond well with results on fungal degradation of thermoplasts, another polluting substrate. In comparative experiments using both, freshwater and seawater media, under atmospheric pressure and under deep sea pressure, we found that the degrading ability of the mycoflora decreased under conditions of increased salinity and hydrostatic pressure (Neumeier *et al.*, 1994; Gonda *et al.*, 2000).

Final comments

Many fungi are known to function well at very low water activities. In addition, fungi generally prefer a slightly acidic pH. Therefore the properties of the Dead Sea - hypersaline, with a pH of about 6, and organic material being available at least during certain periods - would appear suitable for fungal life. It is curious, therefore, that until recently fungi were not taken into account as potential contributors to carbon cycling in the lake. Thus far all heterotrophic activity observed in the Dead Sea was attributed to prokaryotes (Oren, 1988, 1997, 1999, 2000; Oren and Gurevich, 1995). It is too early to claim on the basis of the available data that fungi are an important component of the heterotrophic community in the Dead Sea. However, the finding in the lake of halophilic and/or halotolerant fungi that are able to proliferate in nutrient media containing a high (50% and higher) content of Dead Sea water suggests that the potential for fungal activity in the lake does exist.

The properties of the newly isolated halophilic *Gymnascella marismortui* deserve to be studied in greater depth. It may be especially important to determine its spatial and temporal distribution in the lake in order to decide whether the organism is a real inhabitant of the Dead Sea, or whether its habitat is restricted to the areas of lowered salinity where freshwater from the Einot Zuqim springs mixes with the Dead Sea brines, and/or other areas with a reduced salinity due to freshwater influx.

The main question yet to be answered is to what extent the types of fungi isolated from the Dead Sea occur in the lake as dormant spores only, or also as

vegetative hyphae, potentially contributing to the heterotrophic activity in the lake. It has been claimed that vegetative fungi were found on wooden structures submerged in the Dead Sea water (Buchalo *et al.*, 1998a, 1999, 2000). These fungi need to be isolated and their activity under different salinity regimes has to be studied.

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